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**Characterization and expression of subtilases involved in
grapevine resistance to *Plasmopara viticola***

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Abstract

One of the most important fruit plant cultivated worldwide is grapevine (*Vitis vinifera* L.), mainly due to its economic importance in the wine industry. In 2016 the world area under vines was 7.5 million hectares representing a production over 75.8 million tones, being the European Union the leading producer of wine (OIV, 2017). The domesticated grapevine is highly susceptible to downy mildew caused by the obligatory oomycete *Plasmopara viticola*. This pathogen affects the leaves and shoots, being downy mildew disease characterized by the presence of oil spots on the surface of leaves and white down that can be seen on the underside of the leaves, canes and bunches in periods of high humidity. This disease results in great losses in entire vineyards when control measures are not implemented. The current disease control strategies include the massive use of fungicides, which are very prejudicial to human health. A deeper understanding of the resistance mechanisms is crucial to define alternative control methods.

Subtilisin-like proteases (subtilases) belong to a large group of serine proteases present among all organisms such as archaea, bacteria, eukarya, fungi and yeast. My research group has previously characterized the grapevine subtilase gene family, highlighting the involvement of some subtilases in *P. viticola* resistance. The action mechanisms of subtilases involved in plant defense against pathogens are still unknown; however recent studies have identified prosystemin as the subtilase SBT3 substrate and highlighted the role of the processed systemin in the octadecanoid pathway for jasmonic acid (JA) biosynthesis.

In the present work, we have selected 5 subtilase genes namely, VviSBT3.19 Isoform X2, VviSBT5.3a, VviSBT4.19 Isoform X1, VviSBT3.20, VviSBT3.21 Isoform X1, and analysed their expression in two *Vitis* genotypes (resistant and susceptible to downy mildew), after inoculation with *P. viticola* and elicitation with either JA or salicylic acid (SA). Our results showed that the expression of VviSBT5.3a and VviSBT4.19 increase after both *P. viticola* inoculation and JA elicitation in resistant grapevine genotype in the first hours after inoculation and elicitation. These results suggest subtilases' involvement in the grapevine immunity.

The grapevine subtilase VviSBT4.19 was selected for further functional characterization aiming to unravel its structure and function. The VviSBT4.19 coding sequence was isolated and cloned into both propagation vector (pJET1.2/blunt) and expression vector (pET28a(+)). Two bacteria strains, *E. coli* BL21codon plus and Turner, were tested for recombinant protein production.

VviSBT4.19 expression was tested by induction with IPTG. Although the SDS-PAGE gel did not show a band corresponding to the VviSBT4.19 protein size, a dot blot analysis was performed. Anti-poly-His-tag antibodies were used and a positive result was obtained for IPTG induction. The determination of this subtilase structure will be crucial to understand its importance and function in grapevine resistance against *P. viticola*.

Keywords: *Vitis vinifera* L., *Plasmopara viticola*, VviSBT4.19, recombinant protein expression, elicitation, inoculation, Jasmonic acid

Resumo Alargado

A videira (*Vitis vinifera* L.) é a planta de fruto mais cultivada em todo o mundo devido à sua importância económica na indústria vinícola. A sua área de cultivo mundial atinge os 7,5 milhões de hectares com uma produção de 75,8 milhões de toneladas em 2016, sendo a União Europeia líder na produção mundial de vinho (OIV, 2017). Atualmente, uma das grandes ameaças para a indústria vinícola é o míldio da videira, doença causada pelo oomycete obrigatório *Plasmopara viticola* que afeta todas as castas de *Vitis vinifera* frequentemente usadas na produção de vinho. No seu ciclo de vida, o *P. viticola* hiberna sob a forma de oósporos, estas estruturas são altamente resistentes às condições climáticas adversas, podendo conservar a sua vitalidade durante 2 anos. Sob condições adequadas (temperatura entre os 22-25°C e humidade elevada) os oósporos germinam, dando origem a zoosporângios que libertam zoósporos que germinam e penetram através dos estomas. Na página superior da folha surgem manchas translúcidas e oleosas coincidentes com o aparecimento de um enfeltrado de micélio branco na página inferior levando ao aparecimento de infeções secundárias. Se não forem utilizadas medidas de controlo apropriadas esta doença pode levar a perdas avultadas durante a época de cultivo. Atualmente, as estratégias de controlo da doença assentam exclusivamente na aplicação preventiva de fitoquímicos durante a época de cultivo, acarretando problemas ambientais graves. O conhecimento profundo dos mecanismos de resistência das plantas resistentes a infeção por *Plasmopara viticola* é importante para definir métodos alternativos de controlo.

Em estudos anteriores a interação entre a videira e o *P. viticola* foi caracterizada por uma abordagem de biologia de sistemas. Uma análise detalhada das diferenças entre a resposta de genótipos suscetíveis (interação compatível) e resistentes (interação incompatível) a este patógeno, ao nível da transcritómica, metabolómica e proteómica, permitiu a identificação de mecanismos e de candidatos associados ao estabelecimento da interação incompatível. Um dos candidatos é uma *subtilisin-like protein*, também denominada subtilase. As subtilases são proteases serínicas que exercem funções altamente específicas no desenvolvimento das plantas e em cascatas de sinalização. Na década passada, vários estudos realçaram o papel das subtilases na resposta de defesa a agentes patogénicos nomeadamente no reconhecimento dos efetores dos patógenos, sinalização e ativação de uma resposta imunitária da planta. Apesar do mecanismo de acção das subtilases associadas à resposta de defesa contra patógenos não estar ainda elucidado, estudos recentes em plantas modelo como a *Arabidopsis* e o tomate identificaram a prosistemina (o precursor da sistemina) como o substrato das subtilases durante a ataque por herbívoros e/ou por patógenos. A sistemina é um polipeptídeo com 18 resíduos de aminoácidos ativo presente em concentração muito baixas, que actua na via sinalizadora octadecanóide responsável pela biossíntese do ácido jasmónico (JA). O JA é uma fitohormona vegetal associada a mecanismos de resposta a stress biótico, nomeadamente por patógenos necrotróficos ou herbivoria. Muito recentemente, estudos do nosso grupo de investigação demonstraram o envolvimento do JA na resposta da videira ao patógeno biotrófico *Plasmopara viticola*. A sistemina, como substrato das subtilases foi inicialmente isolada das folhas de tomate *Lycopersicon esculentum* Mill.,

sendo demonstrado que quando uma folha é ferida por herbivoria ou mecanicamente, os genes codificadores de sistemina são expressos, transcritos e rapidamente transportados via floema para outras partes da planta ainda intactas. A sistemina atua sobre cinases proteicas activadas por mitogénio, levando à ativação de fosfolipases e consequente libertação do ácido linoléico das membranas plastidiais. O ácido linoléico é utilizado na síntese do JA que regula, via feedback positivo, a expressão génica da prosistemina. O ácido jasmónico produzido move-se através do sistema vascular onde alcança folhas intactas, desencadeando o processo de defesa. Em estudos anteriores, a família de subtilases de videira foi caracterizada e candidatos putativamente associados à resposta de defesa da videira à infeção com o *Plasmopara viticola* foram identificados. Com o presente trabalho pretende-se validar o envolvimento desses candidatos na resposta de defesa da videira ao *P.viticola*, avaliar a sua relação com a elicitação por fitohormonas (JA e ácido salicílico (SA)), e seleccionar um candidato para validação funcional.

A expressão de 5 genes que codificam as subtilases VviSBT3.19 Isoforma X2, VviSBT5.3a, VviSBT4.19 Isoforma X1, VviSBT3.20, VviSBT3.21 Isoforma X1, previamente identificadas como candidatos associados à resistência da videira ao *P. viticola* foi avaliada por Reação de Polimerização em cadeia em tempo real (qPCR). A sua expressão foi avaliada em dois genótipos de videira, suscetível e resistente ao *P. viticola*, após inoculação com o patogénio e elicitação com JA e SA. A expressão das subtilases VviSBT5.3a e VviSBT4.19 é aumentada nas primeiras horas (6h), tanto após inoculação como também após elicitação por JA no genótipo resistente, sugerindo o seu envolvimento na resposta imunitária da videira ligada à sinalização por JA.

O gene VviSBT4.19 foi seleccionado para uma caracterização molecular e funcional de forma a elucidar a sua estrutura e função na resistência da videira ao *P. viticola*. A região codificante do gene VviSBT4.19 foi amplificada usando oligonucleótidos específicos para a mesma. Posteriormente foi efectuada a clonagem da região codificante desta subtilase num vetor de propagação (pJET1.2/*blunt*) e subsequentemente no vetor de expressão (pET28a(+)). A clonagem e a expressão foram feitas em bactérias adequadas para cada etapa nomeadamente, *E. coli* TOP10 para clonagem, *E. coli* BL21codão+ e *Turner* para expressão. A produção da proteína recombinante foi feita pela adição de β -D-1-tiogalactopiranosideo isopropílico (IPTG). Na análise por eletroforese em gel de poliacrilamida de dodecilsulfato de sódio (SDS-PAGE), não foi possível observar nenhuma banda correspondente à massa molecular da proteína de interesse para a abordagem de indução de expressão aplicada. Para confirmar se o não aparecimento da banda da proteína de interesse no gel de poliacrilamida de dodecilsulfato de sódio era devido a baixa expressão ou ausência da mesma, a expressão da proteína recombinante foi analisada por *dot blot*, uma técnica análoga a *western blot*, utilizando anticorpos anti-caudas de histidina, porque a proteína de interesse estava em fusão com resíduos de histidina na região N-terminal. Duas das cinco bactérias *Turner* induzidas por adição de IPTG foram reconhecidas pelos anticorpos. A determinação da estrutura desta subtilase é fundamental para a compreensão da sua importância e função na resistência da videira ao *P. viticola*.

No momento, estamos a tentar otimizar as condições de expressão e do *western blot* para ultrapassar os problemas ligados a produção da proteína recombinante e interação

entre a mesma e anticorpos, para poder prosseguir com a purificação da mesma, identificação através da espectrofotometria de massa e posterior estudo da atividade enzimática. Se estes problemas de expressão persistirem vamos seguir uma nova abordagem de expressão que consistirá no uso de vetores eucariotas específicos para expressão e sistemas de expressão eucariotas que foram anteriormente descritas para expressão de subtilases de plantas nomeadamente, células de insetos, usadas na expressão da subtilase de tomate LeSBT1 e sistemas de cultura em suspensão usada na expressão da outra subtilase de tomate denominada LeSBT3. Estes sistemas são mais adequados para a expressão de subtilisinas quando comparados aos vetores ou sistemas de origem procariotas.

Palavras Chave: *Vitis vinifera* L., *Plasmopara vitícola*, míldio, inoculação, elicitação expressão, VviSBT4.19 isoforma I.

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Abbreviations

ALE1 abnormal leaf shape 1

bp base pair

CaCl₂ Calcium chloride dihydrate

cDNA Complementary DNA

DAMPs Damage-Associated Molecular Patterns

DNA Deoxyribonucleic acid

DNaseI Deoxyribonuclease I

dNTP Deoxynucleotide

ECL Enhanced chemiluminescence

ECM Extracellular matrix

EDTA Ethylenediaminetetraacetic acid

EF1 α Elongation factor 1-alpha

Fn-III Fibronectin III

gDNA Genomic DNA

hae hours after elicitation

His Histidine

hpi Hours post inoculation

HR Hypersensitive response

H₂O₂ Hydrogen peroxide

IPTG Isopropyl β -D-1-thiogalactopyranoside

JA Jasmonic Acid

KCl Potassium chloride

KH₂PO₄ Potassium dihydrogenphosphate

LB Luria-broth

MAMPs Microbe-Associated Molecular Patterns

MCS Multiple cloning site

MgCl₂ Magnesium chloride

ML Maximum likelihood

mRNA Messenger RNA

Mw Molecular weight

N₂ Nitrogen

NaCl Sodium chloride

Na₂HPO₄ Sodium hydrogenphosphate

OD Optical density

PA Protease-Associated

PAMP Pathogen-Associated Molecular Pattern

PBS-T Phosphate Buffered Saline-Tween

PCR Polymerase chain reaction

PI Proteinase Inhibitors

PR Pathogenesis-related

PRR Pattern Recognition Receptors

PTI Pattern-Triggered Immune response

qPCR Quantitative real time PCR

RNA Ribonucleic acid

ROS Reactive oxygen species

SA Salicylic acid

SDD1 Stomatal Density and Distribution 1

SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Ser Serine

SOB Super Optimal broth

SOC Super Optimal broth with Catabolite repression

TBE Tris-borate-EDTA

TB Terrific Broth

TBS Tris-buffered saline

°C Celsius degrees

g Earth's gravitational acceleration

kDa kilodalton

mg Milligram

mL Millilitre

mM Millimolar

ng Nanogram

nm Nanometre

T_a Annealing temperature

T_m Melting temperature

μL Microlitre

μM Micromolar

1. Introduction

1.1. Grapevine downy mildew

The grapevine (*Vitis vinifera* L.) is one of the most economically important fruit species worldwide (Basheer-Salimia et al., 2014). *Vitis* species belongs to the family Vitaceae, distributed in the temperate zones of the northern hemisphere, with few species reaching the tropics, it comprehends around 60 species, being *Vitis vinifera* L., the domesticated grapevine, the one with most economic importance due to its use in wine production. However, this specie is highly susceptible to several diseases, including downy mildew, one of the most destructive ones. Downy mildew is caused by *Plasmopara viticola* (Berk. & Curt.) Berl. and de Toni affecting shoots, leaves and grapes (Gessler et al., 2011). It is native to the South-eastern United States, and was introduced into Europe in the second half of the nineteenth century (Alleweldt and Possingham, 1988; Gessler et al., 2011). *P. viticola* is an obligate biotrophic oomycete belonging to the family Peronosporaceae (Gindro et al., 2003), it obtains nutrients from living cells of hosts to complete its life cycle through specialized structure known as haustoria (Gessler et al., 2011). *P. viticola* initiates grapevine leaf colonization through motile zoospores that penetrate stomata (Kiefer et al., 2002), after germination, intercellular mycelia growths and form haustoria. In a compatible interaction, haustoria are formed in the first hours after inoculation and in 72hours, intercellular spaces are invaded by mycelium. Finally, sporangiophores emerge through the stomata where they expand into tree-shaped structures carrying the sporangia (Unger et al. 2007). In an incompatible interaction the first infection steps are common, however the infection progress is delayed, inhibited, or completely stopped (Yu et al., 2012).

The majority of the widely grown grapevine cultivars are highly susceptible to *P. viticola* requiring the multiple application of phytochemicals in each growing season, representing major constraints to human health and to the environment. North American *Vitis* species and some Asian *Vitis* species are natural sources of resistance against downy mildew and are used as genetic resources in breeding programs for resistance introgression (Rossetto et al., 2002). Although several resistant hybrids such as Regent, Solaris or Bianca are already successfully established in the market, the development of breeding programs is time consuming and several years may pass until the traits can be observed. So, the search for alternative methods to control grapevine downy mildew is crucial (reviewed by Gessler et al., 2011).

Research is being conducted in order to better characterize the resistance processes in grapevine aiming not only to identify novel genes, proteins or metabolite that may be used for breeding programs but also to develop new disease control strategies that allow a sustainable viticulture. One group of proteins that is gaining particular attention is the serine proteases group (subtilases). There are several evidences that subtilases may be involved in grapevine resistance to *Plasmopara viticola*. The first evidence of the participation of subtilase in the grapevine – *P. viticola* interactions was shown by Figueiredo and co-workers (2008) as while comparing resistant and susceptible genotypes before and after inoculation with this pathogen, where they noticed that the pattern of subtilase expression was increased in resistant genotypes (Figueiredo et al., 2008, 2012, 2016; Monteiro et al., 2013). Other research groups have also described

that when grapevine resistant genotypes were treated with serine protease inhibitors they couldn't overcome pathogen infection becoming susceptible, and susceptible genotypes became even more sensitive (Berger and Altmann, 2000; Gindro et al., 2012; van der Hoorn and Jones, 2004; van der Hoorn, 2008). Gindro and his co-workers discovered that the activation of programmed cell death was one of the main mechanism responsible of overcoming the grapevine infection by *P. viticola* because, when they inhibited the phytaspases, a subgroup of plant subtilases, it increased the infection rate in the resistant and immune varieties, diminished the production of toxic stilbenes and changed the level of the plants susceptibility to the pathogen (Gindro et al., 2012).

Hence, the understanding of the subtilases' role in grapevine resistance mechanisms may contribute to the development of alternative strategies for fungal diseases' control.

1.2. Subtilisin-like proteases (subtilases)

1.2.1. Family characterization

Subtilisin-like proteases (subtilases) belong to a large group of proteases, called serine proteases, that are represented among all groups of organisms such as archaea, bacteria, eukarya, fungi and yeast (Siezen et al., 1991). The subtilase belongs to S8 family which is member of SB clan (Rawlings and Barrett, 1993). Through evolution, many variants of subtilases have arisen and at present can be divided into six main families based on sequence alignment of the catalytic domains (Siezen et al., 2007).

Plant serine proteases are widespread among several taxonomic groups, from trees and crops to legumes and herbs, and although are present in almost all plant parts, seem to be more abundant in fruits (Antão and Malcata, 2005). Unlike mammals on which only nine subtilases have been identified, subtilases from plants are especially abundant. Until present 87 genes were identified in *Vitis vinifera* genome (Figueiredo et al., 2017), 63 genes in *Oryza sativa* (Tripathi and Sowdhamini, 2006), 56 genes in *Arabidopsis thaliana* (Rautengarten et al., 2005), 15 genes in *Lycopersicon esculentum* genome (Meichtry et al., 1999), 23 genes in the moss *Physcomitrella patens*, 90 genes in *Populus trichocarpa* (Schaller et al., 2012), 74 genes encoding 82 potato subtilases (Norero et al., 2016). The majority of plant subtilases are secretory enzymes (Siezen and Leunissen, 1997) that mediate cell-to-cell signalling, stomatal distribution and density control during leaf development (von Groll, 2002), that maintain the shoot apical meristem and the cell wall (Liu et al., 2009; Wolf et al., 2009), process peptide growth factors (Srivastava et al., 2008, 2009), and participate in responses to both biotic and abiotic environmental stressors (Liu et al., 2007; Tornero et al., 1997).

1.2.2. Plant subtilases structure and biochemical properties

The first subtilase structure to be solved was the tomato S1SBT3 by X-ray crystallography (Ottmann et al., 2009) and subtilase function was highlighted (Cedzich et al., 2009; Rose et al., 2009). It was shown that plant subtilases, like those in other organisms, depend on structural elements for the stabilization of the subtilisin domain what may reflect specificity for its roles in physiology of the plant (Rose et al., 2010).

Most subtilases are synthesized as a pre-pro-protein and targeted for secretion by an N-terminal signal peptide. The pro domain is involved in the subtilase maturation through

its cleavage, which is a prerequisite for subtilase passage through the secretory pathway aiming to reach appoplast (Cedzich et al., 2009).

The catalytic triad first observed as Ser–His–Asp in serine proteases composed by serine, histidine and aspartate amino acids is the main characteristic of S8 clade structure because these elements are highly conserved among subtilase family. The structure of this family also usually presents a signal peptide, a pro-domain, a subtilase domain and a protease associated (PA) domain within the subtilase domain (Antão and Malcata, 2005; Dodson and Wlodawer, 1998; Siezen and Leunissen, 1997). Figure 1 represents the conserved structure of plant subtilases but some subtilases present either other domains such as the fibronectin (Fn) III-like domain (Rawling and Salvesen, 2013) or domain deletion.

PA domain in plants is within the S8 peptidase domain. The PA domain in plants is found inserted between the His and Ser active site residues within the subtilase domain (also called S8 peptidase domain), (fig.1), where it causes displacement of the reactive Ser from the catalytic triad to the C-terminal. the PA domain is found in the pyrolysins family of subtilases which includes bacterial endopeptidases, involved in immune response evasion, and plant subtilases such as cucumusin involved in plant pathogen defense and development (Siezen and Leunissen, 1997), and is also implicated in substrate determination of peptidases or from protein–protein interactions (Bruinenberg et al., 1994; Mahon and Bateman, 2000).

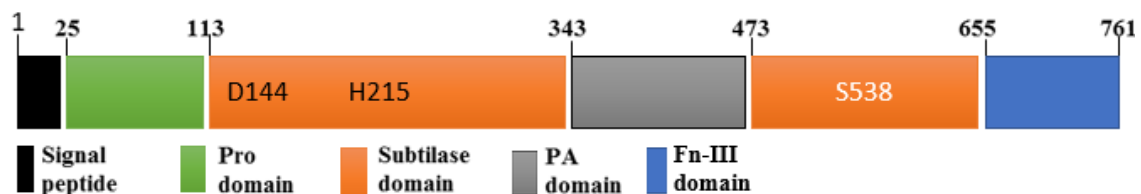


Figure 1: Domain architecture of plant subtilases. presenting all four domains namely, pro-domain, a subtilase domain, a protease associated (PA) domain and the fibronectin (Fn) III-like domain and signal peptide (Adapted from Rose et al., 2010). In addition to the domain borders the figure shows three residues that constitute the active site. This figure represents the domain architecture of S1SBt3.

The additional domain, Fibronectin III (Fn-III), that is present in some subtilases (fig.1), is known to utilize short-peptide surface loops to perform its interactions with other proteins or domain in the signalling pathway cascade (Bencharit et al., 2007). In SBT3, deletion mutants lacking the entire Fn-III domain or part of it were impaired in autocatalytic processing activity and accumulated intracellularly as unprocessed zymogens and the interface of the Fn III domain and the subtilase domain is largely hydrophobic, so it helps the stabilization of the protein by shielding hydrophobic surface patches from solvent. This interaction appears to stabilize the loop system near the active site, what made the researcher consider the Fn-III domain as a required element for SBT3 activity (Cedzich et al., 2009).

The SBT structure is generally described as a monomer (figure 2) suffering a homodimerization through PA domain mediation in order to be activated (Ottmann et

al., 2009; Rose et al., 2010). The PA domain interacts with the pro domain, leading to the cleavage of the N-terminal, and allowing the access of substrates to the catalytic site, for subtilase activity stimulation (Bergeron et al., 2000).

Another feature of subtilases is the apparent Ca^{2+} independence (Rose et al., 2010). The first subtilases were published as enzymes that their activity were influenced by Ca^{2+} presence, because in their structure there are three conserved calcium binding sites and the calcium binding is an important contribution to enzyme stability (Alexander et al., 2001). In contrast to those findings, although Ca^{2+} -binding sites are conserved and critical for stability in other subtilases, SBT3 from tomato was found to be Ca^{2+} -free and its thermostability is Ca^{2+} -independent, because the activity were not influent (Ottmann et al., 2009). For further confirmation Ottmann and other co-workers performed experiment with addition of calcium ions and verified the enzyme activity, surprisingly, no calcium ions could be identified in the structure of SISBT3 despite the fact that the general organization of the calcium binding regions is retained. In another subtilases a positively charged site chain of Lys498 mimics the calcium ion bound function (Rose et al., 2010).

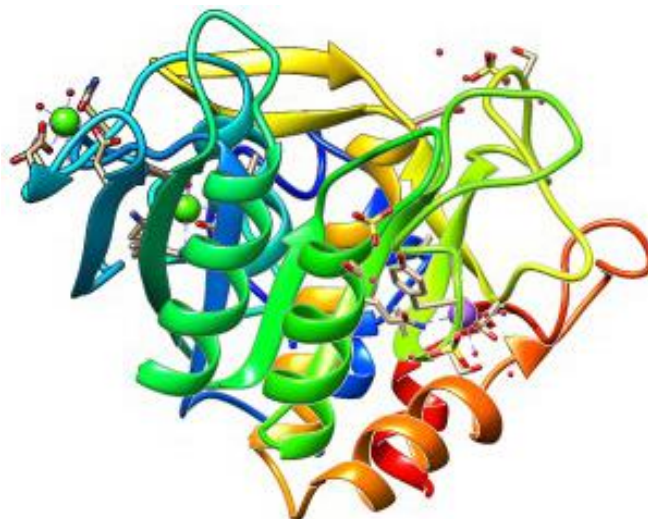


Figure 2: Structure of the SISBT3 monomer. The domains are represented in different colours. Signal peptide correspond to the red sequence, Pro domain in orange, S8 domain in yellow colour, PA domain in green and Fn-III domain in blue colour. This image obtained with the UCSF Chimera package using the following PDB code: 1THM.

1.2.3. Subtilase involvement in plant-specific processes

Plants continuously face biotic and abiotic threats. Subtilases are known to participate, direct or indirectly in most cellular processes, such as, general protein turnover, the cell wall dynamic (either by cleavage of structural protein or by regulation of cell wall remodelling enzymes) (Schaller et al., 2012), specific plant development regulator (Berger and Altmann, 2000) and as a determinant host factor mediating activation of primed immune responses against threat from pathogenic microorganisms (Jones and Dangl, 2006; Ramírez et al., 2013).

Genetics approaches have identified subtilases as highly specific regulators of plant development. For example, in the *Arabidopsis* subtilases, experiments performed that

consist in mutation in SDD1 gene (stomatal density and distribution 1), confer an interruption of stomata formation pattern, resulting in clustering of guard cells and increase of stomatal density (Berger and Altmann, 2000; von Groll, 2002). Another example of gene that was found to encode a subtilase which regulate a plant development is ALE1 (*abnormal leaf shape 1*). This gene is involved in cuticle formation and epidermal differentiation during embryo development in *Arabidopsis* (Tanaka et al., 2001). How do both genes act is the question that is still without answer but it is speculated that these genes are required for the generation of peptide signals, which act non-cell autonomously to control plant development (Berger and Altmann, 2000; Tanaka et al., 2001; von Groll, 2002). The studies by Takeda et al. (2007), highlighted the participation of subtilases in symbiotic interactions. Interaction between plant roots and fungi resulting in a mycorrhiza or between plant roots nitrogen-fixing Rhizobia as nodule symbiosis.

Concerning the interaction between plants microbes, it is known that many plant-associated microbes are pathogens that impair plant growth and reproduction. Plants use two immunological mechanisms to respond to infection namely, innate and acquired immune system. The first one consists of physical and chemical barriers that are dependent on the recognition of broadly conserved molecular features, known as microbe-associated molecular patterns (MAMPs), by plasma membrane proteins known as pattern recognition receptors (PRRs). These receptors also detect a plant degradation product resulting from the action of invading pathogens, or endogenous peptides called damage-associated molecular patterns (DAMPs), and this recognition is called pattern-triggered immune response (PTI) which is characterized by production of reactive oxygen species (ROS), phosphorylation cascades, and a transcriptional reprogramming that lead to defence responses. The second line of protection, adaptive immunity, typically requires activation (Ausubel, 2005; Boller and Felix, 2009; Jones and Dangl, 2006).

In plant immunity, several studies were published in the last decade showing the involvement of subtilases in response to biotic and abiotic environment stimulus. The first evidence of subtilisin-like proteases participation in plant-pathogen interactions was shown by Granell and co-workers when studying induction of pathogenesis-related (PRs) proteins in tomato by citrus exocortis viroid, silver ion and ethephon. After inoculation of tomato with citrus exocortis viroid they identified high levels of P69 (tomato subtilase) (Granell et al., 1987), and two years later it was also implicated in tomato leaves to *Phytophthora infestans* (Christ and Mössinger, 1989), when the researchers were looking for the PRs involved in response *P. infestans* and other biotic and abiotic inducers such as salicylic acid (Jordá et al., 1999) and correlations of those response with resistance. Moreover, during their maturation, the majority of subtilases suffer glycosylation and are secreted to plant extracellular matrix (ECM) where they accumulate and presumably recognize and process substrates (Siezen and Leunissen, 1997; Taylor et al., 1997; Tornero et al., 1996, 1997; Yamagata et al., 1994), so this accumulation of subtilases in ECM of plants rise the possibility for an important subtilase involvement during pathogenesis because, ECM is the place where the first host-pathogen interaction and recognition events take place (Dixon and Lamb, 1990). Later, in *Arabidopsis thaliana* inoculated with oomycete *H. arabidopsidis* and *P. syringae* DC 3000, Ramirez and his co-workers studied an extracellular subtilase switch

for immune priming in *Arabidopsis*. They identified an extracellular subtilase and named SBT3.3, which its loss of function result in an enhanced plant susceptibility, further substantiating its value in establishing an effective plant immune response. They also showed that the production of SBT3.3 rapidly increases during the activation of innate immunity preceding the activation of salicylic acid (SA) responsive genes, responding very rapidly to H₂O₂, a common ROS species generated during PTI to activation of innate immune responses (Ramírez et al., 2013).

1.3. The link between subtilase and phytohormone signaling in grapevine response to *P. viticola*

Little is known about plant subtilases' substrates. Studies in *Arabidopsis* and tomato have identified a prosystemin as the subtilase SBT3 substrate (Bergey et al., 1996; Ryan, 2000). Systemin was the first plant peptide hormone that was isolated from tomato (*Solanum lycopersicum*) by Clarence (Bud) Ryan and his group a quarter of a century ago (reviewed by Pearce et al., 1991). They found out that it was involved with the activation of the systemic wound-induced defense response. Also, it could be several early wound responsive genes (responding within 2–4 h) involved in the octadecanoid pathway for jasmonic acid biosynthesis and of prosystemin (PS) itself or are late (8 h) responsive genes that include genes for proteinase inhibitors (PI-I and PI-II) that interfere with digestive processes in herbivores (Bergey et al., 1996; Ryan, 2000). The prosystemin function was demonstrated with tomato plants transformed with an antisense prosystemin cDNA driven by the constitutive cauliflower mosaic virus promoter. The transformed plants were found to be severely impaired in their systemic wound response and the plants expressing the antisense gene not only accumulated low levels of proteinase inhibitors I and II in leaves of wounded plants, but lost their ability to mount inducible defences against *Manduca sexta* larvae (McGurl et al., 1992, 1994; Orozco-Cardenas et al., 1993).

Bergey and co-workers used the similarity of the structure of jasmonic acid and its precursor phytodienoic acid to the structures of some prostaglandins and the fact that that prostaglandins are derived from arachidonic acid released from membranes by phospholipase A2 as a base to propose a model in which wounding and systemin activated a lipase in receptor cell membranes resulting in the release of linolenic acid, the production of jasmonic acid, and the activation of proteinase inhibitor genes. In that model, oligosaccharides are localized signals, whereas systemin is the systemic signal that activates the defence signalling pathway (Figure 3) (Bergey et al., 1996; Farmer and Ryan, 1992).

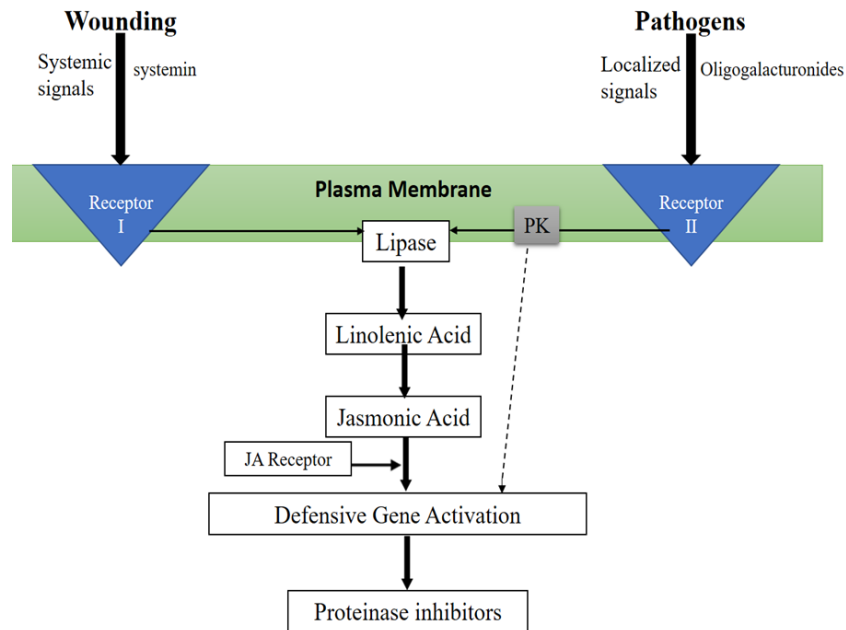


Figure 3: Model for the activation of defensive genes in tomato plants in response to herbivore and pathogen attacks (Adapted from Farmer and Ryan, 1992).

Studies supporting the linking between salicylic acid and jasmonic acid were conducted by Doares and co-workers, where they described that the presence of salicylic acid as inhibitors of the octadecanoid pathway, culminate with deficient plant capacity to respond to a pathogen attack (Doares et al., 1995), by controlling transcriptional reprogramming of JA-induced defensive genes (Caarls et al., 2015). Those results may be related with the reason why one of the subtilase subfamily protease called phytaspase is less expressed or blocked when the octadecanoid pathway for jasmonic acid biosynthesis is inhibited (Beloshistov et al., 2017).

2. Objectives

In grapevine, only recently the participation of subtilases in resistance has been shown. The main goal of this work was to unravel the function of these subtilases and their involvement in grapevine resistance to *P. viticola*, to gain a more comprehensive knowledge on their role in plant immunity, thus contributing to the development of alternative strategies for fungal diseases control.

To achieve this goal the following tasks were performed:

- Phylogenetical analysis of the selected grapevine subtilases (based in the work of Figueiredo et al., 2016) with the subtilases previously described in other plant models as involved in plant immunity and JA signalling;
- Expression analysis of *Vitis* subtilases that share sequence similarity with subtilases predicted to be involved in plant defence against pathogen infection, after inoculation with *P. viticola* and elicitation with jasmonic acid and salicylic acid;
- Selection of resistance and JA-associated candidates;
- Gene isolation, cloning and propagation in adequate expression vectors;
- Recombinant protein production and characterization.

3. Materials and Methods

3.1. Protein Sequence Alignment and Phylogenetic Analysis

Protein sequences from grapevine subtilases putatively involved in grapevine resistance against pathogens (selected in Figueiredo et al. 2017 and Figueiredo J., master thesis, 2016), *Arabidopsis*, tobacco, rice, cotton and tomato subtilases shown to participate in plant immunity were obtained from the NCBI database (February 2017) and aligned using the DNASTAR's MegAlign, version 11.1.0 (59) (<https://www.dnastar.com/t-megalign.aspx>), gaps were manually checked. A maximum likelihood (ML) phylogenetic analysis was performed with MegAlign with the following parameters: protein substitution model PROTCAT; protein substitution model + BLOSUM62; bootstrap 1000 iterations with rapid bootstrap analysis. Tree was viewed and edited on MegAlign (<https://www.dnastar.com/t-megalign.aspx>).

3.2. Plant Material

Two *Vitis vinifera* genotypes, Regent and Trincadeira (tolerant and susceptible, respectively to *P. viticola*) were selected to assess subtilase expression after inoculation with *P. viticola* and elicitation with either jasmonic (JA) or salicylic acids (SA). Plant material inoculated with *P. viticola* and harvested at 6, 12 and 24hpi (Figure 4), as described in Figueiredo et al. (2012) was already available at the laboratory. For the elicitation experiments, wood cutting from the two genotypes were obtained at the Estação Vitivinícola Nacional, at Dois Portos, Torres Vedras, Portugal and grown in 2.5L pots in universal substrate under controlled conditions in a climate chamber (Phytoclimate 5000 EH Aralab, Lisbon) at 23 / 18 °C (day / night), relative humidity 60% and a photosynthetic photon flux density of 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$. One month prior to the elicitation experiments, pots were transferred to the exterior environment at the campus of the Faculty of Science, University of Lisbon (FCUL), Portugal, and irrigated whenever necessary. Grapevine leaves were elicited with 1mM SA (0.2ml) or 1mM JA (0.2mL), (Sigma Aldrich) in 0.05% tween 20 (0.1ml) solutions. Control plants were sprayed with a 0.05% tween 20. The second and third fully expanded leaves beneath the shoot apex were harvested at 6 and 24hours post elicitation (hpe) (Figure 4), immediately frozen in liquid nitrogen and stored at -80°C . Due the damage during the storage, samples collected at 6 hpe with SA, were discarded (Figure 4). Three biological replicates were collected, being each biological replicate a pool of three leaves from three different plants.

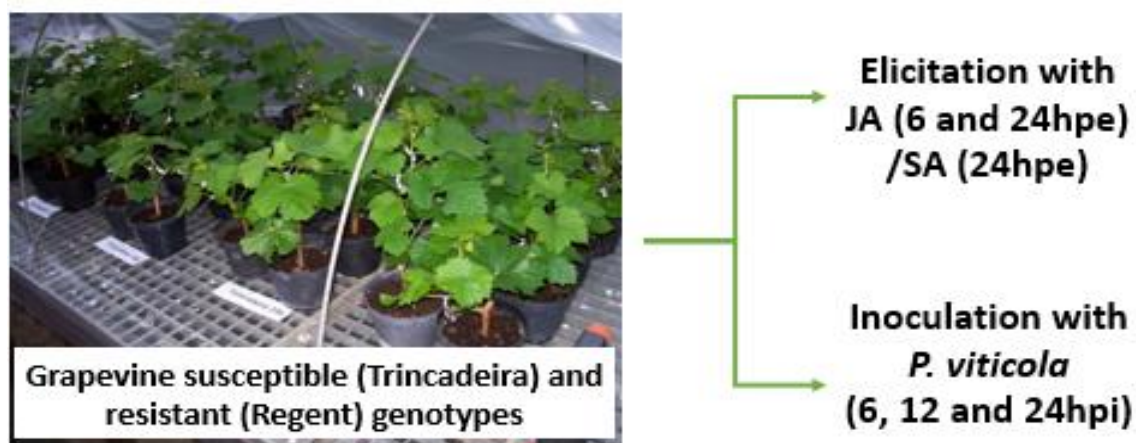


Figure 4: Grapevine genotypes Inoculation with *P. viticola* and Elicitation with JA and SA (Jasmonic Acid and Salicylic Acid). hpe- hours post elicitation, hpi- hour post inoculation.

3.3. RNA Extraction and cDNA Synthesis

Total RNA was isolated from frozen leaves with the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, USA), according to manufacturer's instructions. Residual genomic DNA was digested with DNase I (On-Column DNase I Digestion Set, Sigma-Aldrich, USA). RNA purity and concentration were measured at 260/280nm using a spectrophotometer (NanoDrop-1000, Thermo Scientific) while RNA integrity was verified by agarose gel electrophoresis (1.2% agarose in TBE buffer). Genomic DNA (gDNA) contamination was checked by qPCR analysis of a target on the crude RNA (Vandesompele et al. 2002). Complementary DNA (cDNA) was synthesized from 2.5µg of total RNA using RevertAid®H Minus Reverse Transcriptase (Fermentas, Ontario, Canada) anchored with Oligo(dT)₂₃ primer (Fermentas, Ontario, Canada), according to manufacturer's instructions.

3.4. Quantitative Real Time PCR

Quantitative real time PCR (qPCR) experiments were carried out using Maxima™ SYBR Green qPCR Master Mix (2×) kit (Fermentas, Ontario, Canada) in a StepOne™ Real-Time PCR system (Applied Biosystems, Sourceforge, USA). A final concentration of 2.5mM MgCl₂ and 0.2µM of each primer were used in 25µL volume reactions, together with 4µL of cDNA as template. Primer sequences and reaction details are provided in Table 1. Thermal cycling for all genes started with a denaturation step at 95°C for 10minutes followed by 40 cycles of denaturation at 95°C for 15seconds and annealing at the appropriate temperature (Table 1) for 30seconds. Each set of reactions included a control without cDNA template. Dissociation curves were used to analyse non-specific PCR products (Supplementary data 1). Three biological replicates and two technical replicates were used for each sample. Gene expression (fold change) was calculated as described in Hellemans et al. (2007). The reference genes used for the normalization were the previously described in Monteiro et al. (2013). Statistical significance ($p < 0.05$) of gene expression was determined by the Mann–Whitney U test using IBM® SPSS® Statistics version 23.0 software (SPSS Inc., USA).

Table 1: Candidate reference genes and target genes primer sequences, amplicon length and qPCR analysis. ---- Discarded (low abundance transcript); *The nomenclature used in this table, is in accordance with the last update of *Vitis vinifera* L. subtilases (Figueiredo et al., 2017).

Protein name*/ mRNA Accession Number	Primer sequence	Amplicon length (bp)	Amplification efficiency (E)	Ta (°C)	Tm (°C)	Regression Coefficient (R ²)
Reference genes (Monteiro et al. 2013)						
EF1α (elongation factor 1-α) <i>XM_002284888.2</i>	F: GAAGTGGGTGCTTGATAGGC R: ACCAAAATATCCGAGTAAAGA	164	1.82	60	79.59	0.998
SAND (SAND family protein) <i>XM_002285134.2</i>	F: CAACATCCTTTACCCATTGACAGA R: GCATTGATCCACTTGACAGATAAG	76	1.89	60	78.69	1
Target genes						
VviSBT3.20 <i>XM_002273159.3</i>	F: CAAGCCCCATTAGCACAC R: TTAGAATCAAGATCAAAGAAG	87	----	56	----	----
VviSBT3.19 Isoform X2 <i>XM_003634105.2</i>	F: CCTCCCAATGGAAAAATCTG R: GGCTCATGCTATACAACAAG	170	----	58	----	----
VviSBT5.3a <i>XM_002266692.3</i>	F: CAGCGAGTTTTAGTGATGAAG R: GGGGTATGGAAGGAAGAGT	172	2.08	58	79.77	0.98
VviSBT3.21 Isoform X1 <i>XM_010649370.2</i>	F: GGGATATGGCCTGAGTCTGA R: CAACGCGCACCGATTATTTT	134	2.08	60	79.3	0.93
VviSBT4.19 Isoform X1 <i>XM_010660203.2</i>	F: AATCCTGGTGTCTTGTGG R: ATTAGGTAAATGTTGTGCTTG	73	1.88	56	71.96	0.93

3.5. Cloning of the VviSBT4.19 cDNA

All the steps or procedures followed from the cloning of VviSBT4.19 cDNA to the recombinant protein production are described in the supplementary data 2.

3.5.1. Amplification of VviSBT4.19 Open Reading Frame (ORF)

VviSBT4.19 coding sequence (XM_010660203.2) was amplified with the oligonucleotides VviSBT4.19 forward (5'CCG GAA TTC ATG TGC ATA GCT TAC CTT CTA3') and reverse (5'CAC CGC TCG AGG TGC TTG CCG CAT CAT TTA3'), containing EcoRI and XhoI restriction sites (underlined), respectively. The VviSBT4.19 coding region was amplified by PCR using *Vitis vinifera* cv Regent inoculated with *P. viticola* at 6hpi cDNA as template, with the following program: initial denaturation at 98°C for 30s, denaturation at 98°C for 10s, annealing at 56°C for 30s, extension at 72°C for 2min: 30s, 35 cycles and final extension at 72°C for 10min. The following polymerase chain reaction (PCR) mix was used: 1μl of cDNA, 0.5μl of Phusion polymerase (1.0 unit/50 μl), 2.5μl of primers (10μM), 10μl 5X Phusion HF buffer, 5μl of dNTPs ((10mM) and 28.5μl of nuclease-free water in a total of 50μl. The

PCR products were visualised on a 1.5% (w/v) agarose gel and the band corresponding to the VviSBT4.19 coding sequence was excised and purified using QIAquick PCR Purification Kit Protocol (QIAquick Spin Handbook 03/2008) following manufacturer's instructions. Concentration was measured in a NanoDrop 1000 (Thermo Scientific).

3.5.2. Bacterial strains, and vectors

E. coli TOP10 (Invitrogen, New York) and pJET1.2/blunt Cloning vector (Thermo Scientific, Waltham, Massachusetts, USA) (Figure 4A) were used to perform DNA manipulation. *E. coli* BL21codon plus (Stratagene, California, USA) and pET28a(+) (Novagen, USA) (figure 4B), were used for *Vitis vinifera* Cucumisin (VviSBT4.19) gene expression.

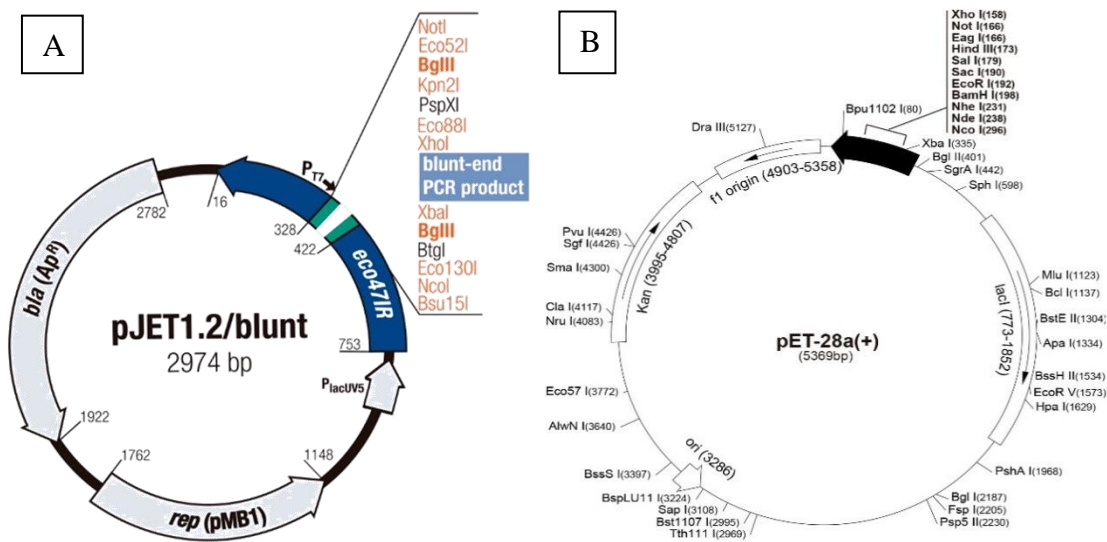


Figure 5: Schematic maps of vector used for VviSBT4.19 cloning and expression. A- pJET1.2/blunt Cloning vector contain β -lactamase gene conferring resistance to ampicillin, Lethal gene *eco47IR* which enables positive selection of the recombinants, T7 RNA polymerase promoter for in vitro transcription of the cloned insert, Multiple cloning site (MCS), Insertion site Blunt DNA ends for ligation with insert. B- This vector (pET-28a(+)) contain a T7 promoter, a T7 terminator, a T7 transcription start region, His-tag coding sequences, a multiple cloning site, coding sequence of gene *lacI*, a pBR322 origin of replication and kanamycin resistance gene and a f1 origin of replication.

3.5.3. VviSBT4.19 cloning in pJET1.2/blunt Vector

To construct the recombinant plasmids, VviSBT4.19 gene was cloned into the pJET1.2/blunt vector using CloneJET™ PCR Cloning Kit (#K1231, #K1232), according manufacturer's instructions, under following volumes: 6 μ L of nuclease-free water, 10 μ L of 2X reaction Buffer, 1 μ L pJET1.2/blunt of 2 μ L of the gene and 1 μ L of T4 DNA Ligase. The ligation was performed at 22°C for 5min.

3.5.4. Preparation of chemically competent *E. coli* One Shot TOP10 cells

E. coli One Shot TOP10 was submitted to a protocol for competence induction and used as host for amplification of recombinant plasmids. Cells were plated in SOB (Super Optimal Broth) medium at 37°C overnight. One colony was inoculated in 225mL of SOB medium and grown at 37°C, 170 rpm until the OD_{600nm} reached 0.5. Cells were kept on ice for 10 minutes and centrifuged at 1400g for 5minutes. The supernatant was discarded and the pellet resuspended in RF1 buffer (100mM RbCl₂, 50mM MgCl₂ (4H₂O), 30mM KAC, 10mM CaCl₂·2H₂O, 15% (v/v) glycerol). Cells were kept on ice for 15minutes and centrifuged at 1400g for 5minutes. The supernatant was discarded and the pellet re-suspended in RF2 buffer (100mM MOPS, 10mM RbCl₂, 75mM CaCl₂·2H₂O, 15% (v/v) glycerol). Cells were divided in 100μL aliquots and frozen in liquid N₂.

3.5.5. *E. coli* One Shot TOP10 transformation

Competent *E. coli* One Shot TOP10 cells were transformed with the pJET-VviSBT4.19 constructs according following procedures: 100μl of cells were thawed on ice and 10μl of ligation product was added and incubated on ice for 30 minutes, also submitted to a heat-shock for 45seconds at 42°C, without shaking, and re-incubated on ice for 2minutes. 800μL of LB culture medium was added and incubated at 37°C, 200rpm for 30minutes. Cells were centrifuged at 1200g for 2minutes at room temperature and the supernatant was discarded. The cell pellet was re-suspended in remaining medium and plated in LB agar medium supplemented with 50μg/mL of ampicillin for growth at 37°C overnight.

3.5.6. Colony PCR and pJET-VviSBT4.19 purification

The presence of the VviSBT4.19 was confirmed by colony PCR using the cell colonies grown in the agar plate as template. Colony PCR reaction conditions were: initial denaturation at 95°C for 3min, denaturation at 94°C for 30s, annealing at 60°C for 30s, extension at 72°C for 2min:30s, 35 cycles and final extension at 72°C for 10min. The volumes of PCR components of 20μl contained 0.25μl of GoTaq polymerase (5U/μl), 0.4μl of each primer (10μM), 4μl 5X Colorless GoTaq® Reaction Buffer, 2μl of dNTPs (10mM), 1.2μl of MgCl₂ (1.5mM) and 11.75μl of nuclease-free water and one colony were added in each mix. The colony PCR products were analysed in 1% agarose gel electrophoresis. Bacteria presenting the PCR band correspondent to the VviSBT4.19 insertion were inoculated in LB medium supplemented with 50μg/mL of ampicillin and incubated at 37°C, 200rpm, overnight. The constructs were extracted using NZYMiniprep Kit, according to manufacturer's instructions. The purified products were quantified in a NanoDrop 1000 (Thermo Scientific). For further confirmation of the presence of the pET28a(+) vector and the gene, the purified plasmid were digested using EcoRI and XhoI restriction enzymes (Thermo Scientific, Waltham, Massachusetts, EUA) under following conditions: 10μL of nuclease-free water, 8μL of 10X Tango buffer, 20μL of pET28a(+) or pJET-VviSBT4.19, 1μL of each enzyme. The reaction occurred at 37°C for 2 h and the enzymes were inactivated at 80°C for 20min,

with subsequent analysis by 1.0% (w/v) agarose gel electrophoresis and analysed by gene sequencing using the T7 primers (STABVida Company, Caparica, Portugal)

3.5.7. Cloning in the expression vector pET28a+

The plasmid containing the VviSBT4.19 insertion was extracted from *E. coli* One Shot TOP10 using the NZYMiniprep Kit, per manufacturer's instructions. Both plasmid containing the VviSBT4.19 and pET28a(+) were hydrolysed using EcoRI and XhoI restriction enzymes as previously described.

The VviSBT4.19 ORF was cloned into the expression vector (pET28a(+)) using the T4 DNA Ligase enzyme (New England Biolabs) as follows: 9µL of nuclease-free water, 2µL of 10X T4 DNA Ligase Buffer, 2µL of pET28a(+), 6µL of VviSBT4.19 and 1µL of T4 DNA Ligase. The ligation was performed at 22°C for 2hours. The plasmid and gene volume were calculated using the formula below:

$$ng\ insert = \frac{bp\ insert}{bp\ vector} \times 3 \times ng\ vector$$

The ligation product was used to transform *E. coli* BL21 and One Shot TOP10 cells using same procedures as described above.

3.5.8. Colony PCR and pET28a(+)-VviSBT4.19 purification

The transgene insertion was confirmed by colony PCR as previously described. The colony PCR products were analysed in 1% (w/v) agarose gel electrophoresis. The recombinant bacteria were inoculated in LB medium supplemented with 50µg/mL of kanamycin and incubated at 37°C, 200rpm, overnight. The constructs were extracted from bacteria using NZYMiniprep Kit, according to manufacturer's instructions. To further confirm the presence of the pET28a(+)-VviSBT4.19, the purified DNA were digested using EcoRI and XhoI restriction enzymes, with subsequent analysis by 1.0% (w/v) agarose gel electrophoresis, as previously described. Gene sequencing using the T7 promotor and T7 terminator primers was also performed (STABVida Company, Caparica, Portugal).

3.6. Expression of VviSBT4.19 in BL21 Codon Plus and Tuner bacteria

3.6.1. Competent *E. coli* BL21-CodonPlus and Tuner cells (Stratagene) preparation

E. coli BL21-CodonPlus (Stratagene) are engineered to contain extra copies of genes that encode the tRNAs that most frequently limit translation of heterologous proteins. *E. coli*. Tuner (Novagen, USA) contains a mutation in the lac permease (lacZY) gene. This enables adjustable levels of protein expression throughout all cells in a culture. The lac permease (lacY) mutation allows uniform entry of IPTG into all cells in the population, which produces a concentration-dependent, homogeneous level of induction. By

adjusting the concentration of IPTG, expression can be regulated from very low levels up to the robust, fully induced levels commonly associated with pET vectors.

E. coli BL21-CodonPlus and Tuner cells were submitted to protocol of Cohen *et al.*, 1972 for competence induction and used as host for expression of recombinant plasmids. Cells were plated in solid LB (Luria-Broth) medium supplemented with 34µg/mL chloramphenicol at 37°C overnight. One colony was inoculated in 3mL of liquid LB medium supplemented with 34µg/mL chloramphenicol at 37°C overnight, with shaking (250 rpm). About 500µL of cells were inoculated in 10mL of liquid LB medium supplemented with 34µg/mL chloramphenicol and incubated at 37°C, 200rpm for 3h. Cells were centrifuged at 1500g for 7minutes. The supernatant was discarded and the pellet re-suspended in 3ml CaCl₂, also incubated on ice for 20minutes and centrifuged at 1500g for 7minutes. The supernatant was discarded, the pellet re-suspended in 1ml CaCl₂ and kept on ice (1h minimum) until using for transformation.

3.6.2. Transformation of expression bacteria (BL21-CodonPlus and Tuner)

The extracted and purified positive constructs (pET28a(+)-VviSBT4.19) were used to perform the transformation of BL21-CodonPlus cells, described as a member of large group of expression bacteria.

Competent *E. coli* BL21-CodonPlus and Tuner cells were transformed using following procedures: 100µl of cells were thawed one ice and 100ng of pET28a(+)-VviSBT4.19 were added and incubated on ice for 30minutes, and then submitted to a heat-shock for 2 minutes at 42°C, without shaking, and re-incubated on ice for 3minutes. About 800µL of LB culture medium was added and incubated at 37 °C, 200 rpm for 1h. Cells were centrifuged at 1200g for 2minutes at room temperature and the supernatant was discarded. The cell pellet was re-suspended in remaining medium and plated in LB agar medium supplemented with 50µg/mL of kanamycin and chloramphenicol for growth at 37°C overnight. The recombinant cells presence was confirmed by colony PCR and miniprep using the cell colonies grown in the agar plate following same condition as described above.

3.6.3. Recombinant protein expression in a pET28a(+) vector in the BL21-CodonPlus cells and inTuner cells

Recombinant colonies were grown overnight in TB medium (3ml) at 37°C with shaking. The overnight culture was transferred into TB medium (10ml) containing kanamycin (50 µg/ml). The culture was incubated at 37°C until an OD_{600nm} of 0.5-0.6 was reached. IPTG (0.1 mM) was added to induce expression and the culture was incubated for 4h at 37°C. The culture was centrifuged (2000 g, 10min, 4 °C), the pellet resuspended in 20ml of PBS-T [137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.76 mM KH₂PO₄, 0.1% (v/v) Tween-20]. The suspension was incubated for 10min at RT and stored at -20°C. After thawing, cells were disrupted by sonication on ice for 4x20s with 10s intervals and centrifuged (5000g, 10min, 4°C). The supernatant was filtered

through. To assess the expression and solubility of the protein, the supernatant and pellet of recombinant cells were analysed by 10% SDS-PAGE.

3.6.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blot

Protein samples were separated in a 10% reducing SDS-PAGE (using a 4% stacking gel) as follows: samples (10 μ l) were combined with an equal volume of loading buffer [125mM Tris-HCl buffer, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.02% (v/v) bromophenol blue], were heated for 5min and placed on ice until loaded into the gel. After sample loading, electrophoresis was conducted at 90V for 90min. The gel was stained with Coomassie blue staining solution [0.2% (w/v) Coomassie blue, 7.5% (v/v) acetic acid, 50% (v/v) ethanol] for 2h. Coomassie stained gels were destained with destaining solution [50% (v/v) methanol, 10% (v/v) acetic acid] for 2 h. The image was captured on Gel Doc™ XR+ Gel Documentation System.

For western blot analysis, the Mini Trans-Blot cell (Biorad, California-EUA) was used, following the manufacturer's instructions. To perform the blotting, a PVDF membrane previously activated in 100% methanol was used. Transfer buffer contained 3.0g/l Tris, 14.4 g/l glycine, 20% (v/v) methanol. Transfer was conducted at 90V in blotting apparatus for 70min. After protein transfer, the membrane was blocked for 1h at RT with 3% (w/v) non-fat milk in TBS-T (20mM Tris-HCl buffer, 200mM NaCl, 0.1% Tween-20, pH 7.4). The membrane was incubated overnight, at 4°C, with mouse anti-Histag primary antibody (His-probe Antibody (H-3), Santa Cruz Biotechnology, INC) diluted 1:1000 in 0.5% (w/v) TBS-T. The membrane was washed (3x10min) in TBS-T and then incubated 1h, RT, with HRP-linked secondary antibody (m-IgG κ BP-HRP) diluted 1:1000 in 3% (w/v) TBS-T, and then washed with TBS-T (3x10min). Membranes were incubated in ECL Western Blotting Detection Reagent in the dark before digital imaging which is also performed in the dark.

3.6.5. Dot Blot

The PVDF membrane was activated in 100% methanol for 5min. The membrane and filter paper were soaked into transfer buffer (3.0g/l Tris, 14.4g/l glycine, 20% (v/v) methanol) for 5min. The filter papers were placed on a clean surface and the membrane was placed on the filter paper, to guarantee the capillarity. The boiled samples (the lysates combined with an equal volume of loading buffer [125 mM Tris-HCl buffer, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol]) were applied on the membrane before it dries. The subsequent steps were the same as for the western blot, which include the blocking and the incubation with primary and secondary antibodies and digital imaging.

4. Result and discussion

4.1. Phylogenetical analysis of selected grapevine subtilases

Plants continuously face threats from quite a diversity of sources. Nowadays, there is evidence of direct or indirect participation of subtilases in the plant responses to these threats, for example as a determinant host factor mediating activation of primed immune responses against pathogenic microorganisms (Jones and Dangl, 2006; Ramírez et al., 2013). Duan and co-workers used yeast two-hybrid assay and bimolecular fluorescence complementation (BiFC) analysis to show that the cotton subtilase GbSBT1 interacts with a prohibitin (PHB)-like protein expressed in *V. dahliae* pathogens during infection (Duan et al., 2016). Previous studies done by Ramírez and co-workers in *A. thaliana* also associated subtilase AtSBT3.3 with the defence response to pathogen attack (Ramírez et al., 2013) and same results were found in P69 in *S. lycopersicum* (Tornero et al., 1996). In grapevine, following the characterization of the grapevine subtilase gene family by our group, 13 subtilases were associated with *Plasmopara viticola* resistance (Figueiredo et al., 2016). In order to understand if these 13 grapevine subtilases are related to other plant subtilases described as participating in immune priming, plant resistance and JA signalling, a phylogenetical analysis was performed. The subtilase sequences GbSBT1 from cotton (Duan et al., 2016), P69B, P69C and SISBT3 from tomato (Meichtry et al., 1999), phytaspase from tobacco (Chichkova et al., 2010), tomato (Beloshistov et al., 2017) and rice (Tripathi and Sowdhamini, 2006), StSBTc from *Solanum tuberosum* (Fernández et al., 2015), and AtSBT3.3, AtSBT5.2(b) and AtSBT6.1 from *Arabidopsis* (Rautengarten et al., 2005) were used as resistance-associated subtilases.

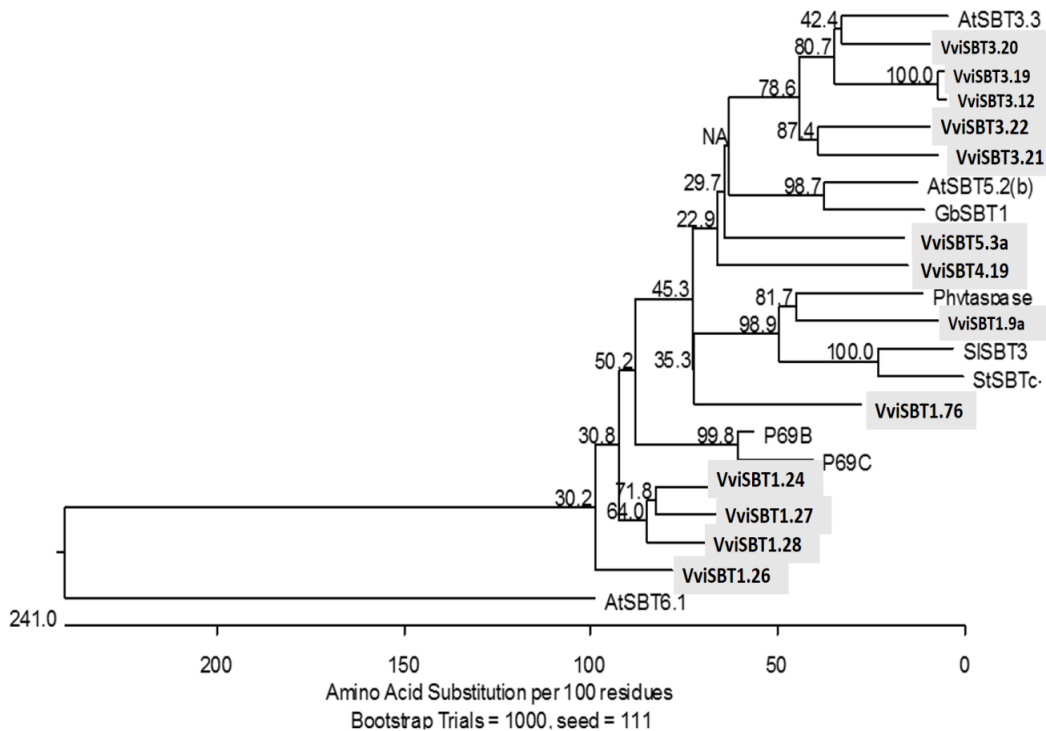


Figure 6: Phylogenetical analysis of 13 *Vitis* subtilases with other plant subtilases predicted to be involved in plant defence against pathogen infection. VviSBT3.20, VviSBT3.19 Isoform X2, VviSBT3.12 Isoform X1, VviSBT3.22, VviSBT3.21 Isoform X1, VviSBT5.3a, VviSBT4.19 Isoform X1, VviSBT1.9a, VviSBT1.76, VviSBT1.24, VviSBT1.27, VviSBT1.28 and VviSBT1.26 are the selected 13

Vitis subtilases; SISBT3, P69B and P69C are tomato subtilases; *Phytaspase* is subtilase from tobacco, StSBTc the *Solanum tuberosum* subtilase; AtSBT3.3, AtSBT5.2(b) and AtSBT6.1 are *Arabidopsis* subtilases; GbSBT1 is a cotton subtilase.

Seven grapevine SBT Protein sequences VviSBT3.19 Isoform X2, VviSBT5.3a, VviSBT4.19 Isoform X1, VviSBT3.20, VviSBT3.21 Isoform X1, VviSBT3.12 Isoform X1, and VviSBT3.22 showed sequence similarity to the immunity related subtilases namely, cotton and *Arabidopsis* subtilase (Figure 5). In the phylogenetical analysis VviSBT3.19 Isoform X2 and VviSBT3.12 Isoform X1 are closely related (fig.5), the same occurs with VviSBT3.21 Isoform X1 and VviSBT3.22, thus VviSBT3.19 Isoform X2 and VviSBT3.21 Isoform X1 were selected for further analysis.

In summary, 5 grapevine subtilases were chosen (VviSBT3.19 Isoform X2, VviSBT5.3a, VviSBT4.19 Isoform X1, VviSBT3.20, VviSBT3.21 Isoform X1) for gene expression profiling after inoculation with *P. viticola* and elicitation with either JA or SA.

4.2. Expression analysis of the selected grapevine subtilases after inoculation with *P. viticola* and elicitation with JA or SA

We have evaluated by qPCR the expression profiling of the selected grapevine subtilases upon both pathogen inoculation and JA or salicylic acid elicitation in two grapevine genotypes: *Vitis vinifera* cv Regent and cv Trincadeira, resistant and susceptible to *P. viticola* respectively. We have chosen these phytohormone to elicitate these cultivars because Guerreiro et al.(2016), related the activation of JA and SA pathway at early stages (6, 12, 24hpi) of inoculation with *P. viticola*. The employment of synergistic/antagonistic mechanisms may a boost to have deeper understanding of *V. vinifera* cv. Regent response to the biotrophic oomycete *P. viticola*.

Two (VviSBT3.19 and VviSBT3.20) of the 5 subtilases analysed did not show any results, so they were discarded due low abundance transcript (table 1). After *P. viticola* inoculation, in the susceptible genotype, the expression of VviSBT5.3a decreased at 6hpi while the expression of VviSBT3.21 increased. VviSBT4.19 showed no alteration. Contrarily, the resistant genotype VviSBT5.3a, VviSBT4.19 and VviSBT3.21 were highly upregulated with the higher expression increase being of the subtilase VviSBT4.19 (figure 6A).

Cao and co-workers (2014) analysed the expression of several grapevine subtilases under different abiotic stimuli and in different tissues without stimulation. They found that the expression of the subtilase VviSBT3.21 was suppressed under abiotic stress conditions. Figueiredo and co-workers, studying the cultivar-specific kinetics of gene induction during downy mildew early infection in grapevine, showed an expression increase of VviSBT3.21 at 6hpi (Figueiredo et al., 2012). This result suggests that this subtilase may be involved in the response to biotic stimulus, but not in response against abiotic stimulus.

At 12hpi all 3 subtilases were upregulated in the susceptible genotype but the VviSBT4.19 showed high level of expression comparatively to VviSBT5.3a and VviSBT3.21. In the resistant genotype, both VviSBT5.3a and VviSBT3.21 were

downregulated and in contrast VviSBT4.19 was upregulated. At 24hpi all 3 subtilases were upregulated in the susceptible genotype and resistant genotype, but with decreased fold change comparatively to 12hpi time point in the resistant genotype (figure 6A). This regulation may lead to the hypothesis that these subtilases may be participating in the early stage of pathogen infection in the resistant genotype and that a time-delay is occurring in the susceptible genotype. This delay in the response of grapevine susceptible genotypes may be associated to an attempt to establish a defense response that is not strong or fast enough to overcome the pathogen, as already described by Figueiredo and co-workers (2012). Moreover, it is speculated that these proteins may be playing an important role in the resistant cultivar once they may be participating in the regulation of biological processes such as pathogen recognition leading to further induction of defence responses (Jordá et al., 1999; Tornero et al., 1996, 1997; van der Hoorn and Jones, 2004).

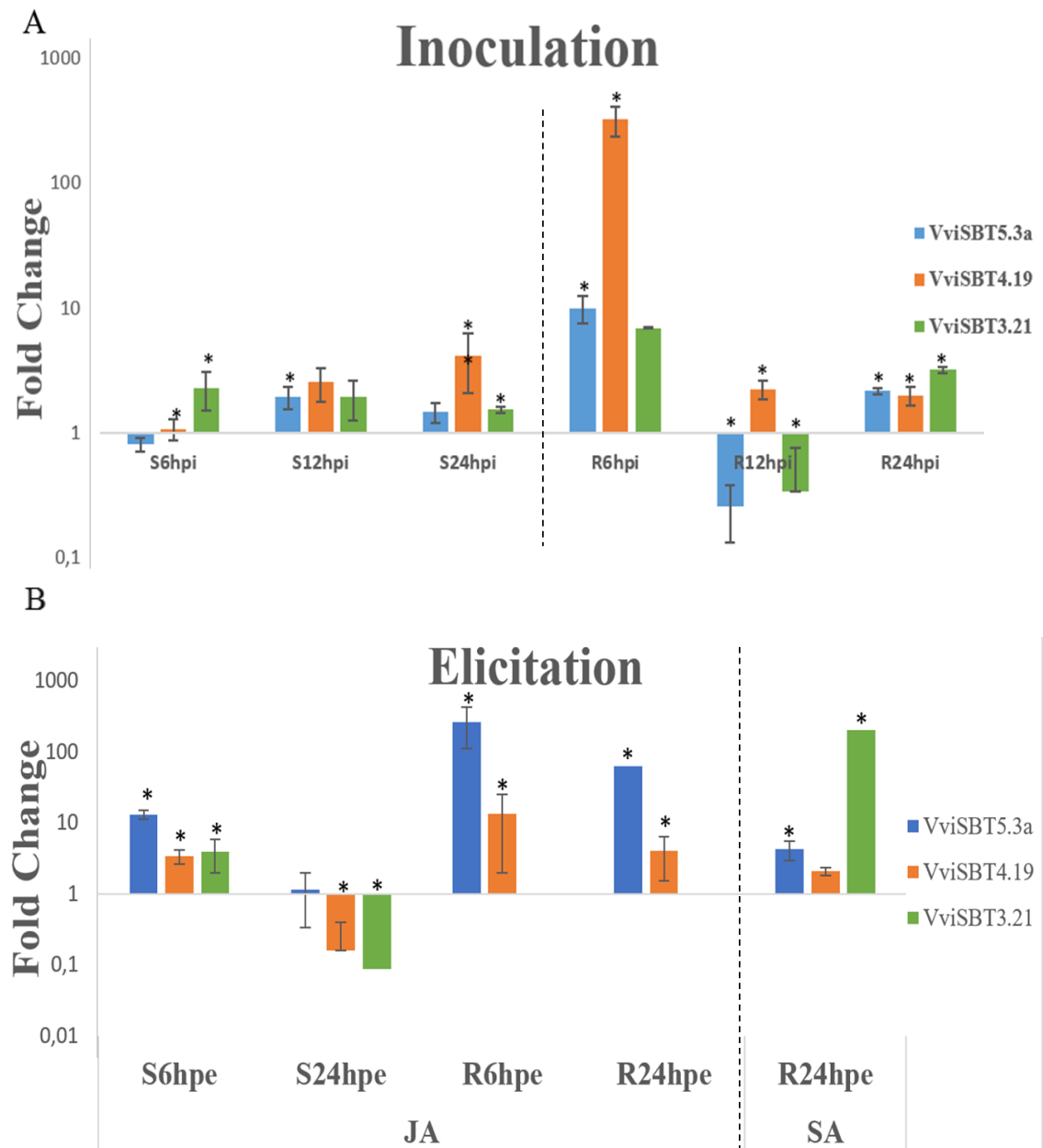


Figure 7: Subtilases expression profile in *Vitis* cultivars after inoculation with *P. viticola* (A) and elicitation with JA or SA (B). Values between 0 and 1 correspond to gene down-regulation, values around 1 mean basal expression and values higher than 1 indicate up-regulation. S6hpi, S12hpi, S24hpi, R6hpi, R12hpi, R24hpi correspond to the time-points after inoculation of susceptible (S) and resistant (R) cultivars. S6hJA, S24hJA, R6hJA, R24hJA R24hSA correspond to the time-points after elicitation of susceptible (S) and resistant (R) cultivars. S-susceptible *V. vinifera* cultivar (Trincadeira), R-resistant *V. vinifera* cultivar (Regent), JA-jasmonic acid and SA-salicylic acid. Asterisks (*) represent significant difference ($p \leq 0.05$) between target and control samples (Mann Whitney U test; SPSS Inc., USA, V20).

Concerning the elicitation experiment, at 6 hae the subtilases VviSBT5.3a, VviSBT4.19 and VviSBT3.21 were upregulated in the susceptible genotype whereas in the resistant genotype both VviSBT5.3a and VviSBT4.19 were upregulated; VviSBT3.21 did not respond to JA elicitation at this time-point (Figure 6B). It suggested that VviSBT3.21 is not quite correlated with the grapevine defence against pathogen attack, as we had seen

in the in the inoculation experiment, contrarily to VviSBT5.3a and VviSBT4.19 which are consistent with the inoculation experiment.

At 24 hae with JA, VviSBT5.3a showed basal expression and both VviSBT4.19 and VviSBT3.21 were downregulated in the susceptible genotype. In the resistant genotype, both VviSBT5.3a and VviSBT4.19 were upregulated, contrarily to these two subtilase, VviSBT3.21 didn't respond to JA elicitation.

Bergey et al. (1996) and Ryan (2000) studying the solanaceae wound response, showed that the activation of defensive genes through the pro-systemin (PS) associated to the octadecanoid pathway activation occurs between 2-4hae inducing genes with digestive processes in herbivores. The grapevine subtilases VviSBT5.3a and VviSBT4.19 may be also participating in the activation of the octadecanoid pathway as their expression is responsive to JA elicitation.

After elicitation with SA (24hae), VviSBT5.3a and VviSBT4.19 expression decreased, while VviSBT3.21 increased its expression profile (Figure 6B), suggesting that the first 2 subtilases were inhibited by SA and VviSBT3.21 was stimulated in the SA presence. Schaller and co-workers investigated the role of SA in induction of defensive genes expression by inhibition of SA biosynthesis (Schaller et al., 2000). After potato plants' treatment with fusicoccin (FC), a toxin produced by the fungus *Fusicoccum amygdali*, the accumulation of defensive genes transcripts was found to be less expressed or blocked in the presence of SA, greatly enhanced in the presence of SA inhibitor, which means that SA is not necessary for the expression of intracellular PR proteins (Schaller et al., 2000), and it also appears to inhibit the accumulation of these transcripts (Niki et al., 1998). This means that SA acts as an inhibitor of JA signalling pathway (Caarls et al., 2015). Our results suggested that VviSBT5.3a and VviSBT4.19 are involved in the JA biosynthesis pathway, being this the reason why they decreased their expression in the presence of SA, when the JA pathway was blocked.

4.3. Cloning of VviSBT4.19 coding sequence

The expression profile analysis of the selected grapevine subtilases showed that, when submitted to biotic (*P. viticola*) or phytohormone (JA and SA) stimulus both VviSBT5.3a and VviSBT4.19 increase their expression in the resistant grapevine genotype. Hence, the VviSBT4.19 subtilase was further selected for functional characterization as its expression was highly increased by both *P. viticola* and JA in the resistant genotype (Figure 6A). This subtilase was also previously identified as a resistance-associated candidate in the grapevine-*P. viticola* pathosystem (Figueiredo et al., 2008, 2012).

Vitis vinifera cDNA was used as a template for PCR using primers designed from the VviSBT4.19 coding sequence (XM_010660203.2) as shown in supplementar data 3. The obtained PCR product, of about 2400bp, is shown in fig. 7.I (lane D). The observed fragment length is consistent with the size of 2360bp predicted for the VviSBT4.19 fragment, to which was added the restriction sites born by the primers. The PCR product was then excised and purified from the gel, ligated into the pJET1.2/blunt vector, and used to transform *E. coli* One Shot TOP10 cells. The presence of

recombinant colonies was confirmed by colony PCR using T7 primers. Product is shown in Figure 7.II (lane L), with an approximate size of 2500bp, as expected.

Recombinant vectors were isolated from transformed cells. As shown in Figure 7.I (lane C), double restriction of recombinant vectors with EcoRI and XhoI released fragments of estimated size of 5400pb, 3000 bp and 2400bp, corresponding to the pJET1.2-VviSBT4.19 construct, pJET1.2 vector and VviSBT4.19 fragments respectively. The size of the pJET1.2 (Figure 7.I. lane B) is estimated to be 2974 bp.

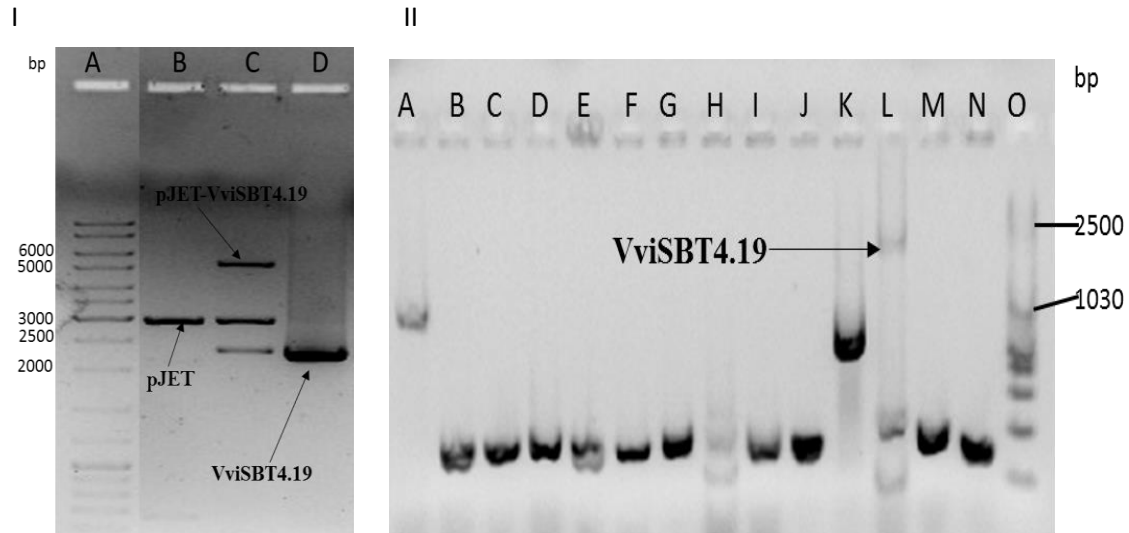


Figure 7: Agarose gel analysis of amplification of VviSBT4.19 using *Vitis vinifera* cDNA as template and VviSBT4.19 cloning into pJET vector. (I) DNA molecular weight marker (lane A); pJET vector as a positive control (lane B); partial double restriction of recombinant plasmid (lane C) and amplified VviSBT4.19 (lane D). (II) Positive colony (lane L); DNA molecular weight marker (lane O); negative colonies (from lane A-N excluding lane L). Arrows indicate the position of the VviSBT4.19, pJET and pJET1.2- VviSBT4.19 construct fragment.

The fragment of interest (2360bp) shown in Figure 7.I (lane C) was excised and purified from the gel and visualised on an agarose gel, before being sub-cloned into bacterial expression vector [pET28a(+)]. Recombinant colonies were screened by double restriction with EcoRI and XhoI. All 5 screened colonies for vector pET28a(+) were recombinant, as shown by the presence of 5400bp and 2400bp fragments (Figure 8), corresponding to the pET28a(+) vector and VviSBT4.19 fragments respectively. The size of the pET28a(+) (lane B-F) is estimated to be 5360bp.

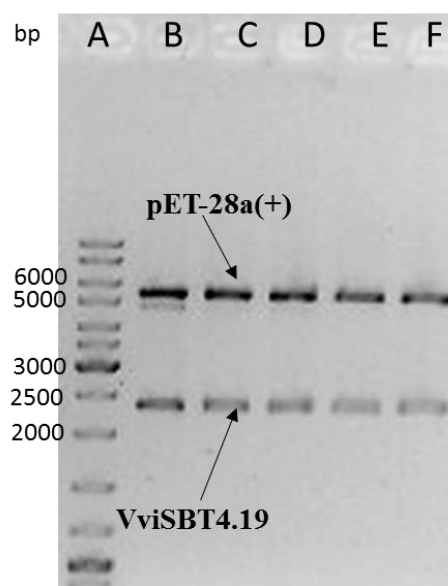


Figure 8: Agarose gel analysis of VviSBT4.19 sub-cloning into bacterial expression vector [pET28a(+)]. DNA molecular weight marker (lane A) and from lane B to F are the results of double restriction of pET28a(+)-VviSBT4.19 constructs. Arrows indicate the position of the VviSBT4.19 and pET28a(+) fragments.

4.4. Recombinant VviSBT4.19 protein expression in a pET28a(+) vector

All 5 positive colonies were submitted to expression protocol, where each of them were induced by the addition of IPTG. Using these approaches, the SDS-PAGE gel did not show a band corresponding to the VviSBT4.19 protein size, which is 79kDa plus the 6kDa his-tag, resulting in a fusion protein with 85kDa.

For further confirmation of the lower expression or absence of protein expression we performed a western blot using an anti-his-tag e started by analysing the protein extracts by Dot blot, which is easier and quicker to perform than western blot. Crude lysates were probed with anti-poly-His-tag antibody, where only two of the five Turner *E. coli* cells lysate, carrying recombinant pET28a-VviSBT4.19, (expression induced by IPTG), were recognised by anti poly-His-tag antibody (Figure 9). The antibody did not recognise B121 codon plus *E. coli* cell lysates carrying recombinant pET28a-VviSBT4.19.

To understand, whether these blots correspond to our fusion VviSBT4.19 protein we performed a western blotting. However, the anti-poly-His-tag antibody did not recognise any of the lysates. The western blot conditions were further optimized, by increasing the antibody concentration and incubation time, and also the amount of protein loaded in the gel. Nevertheless, no signal was detected in the blot. The protein transfer to the membrane was confirmed by Ponceau S staining.

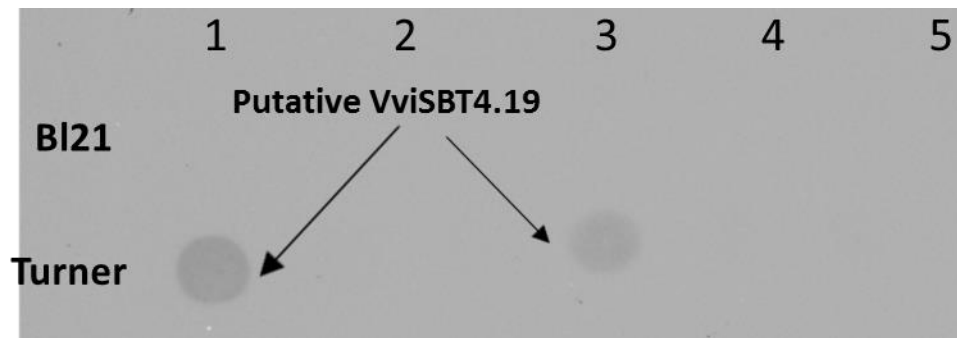


Fig. 9: **Dot blotting analysis of expressed VviSBT4.19 fusion proteins.** Arrows indicate the position of putative fusion VviSBT4.19 proteins. Arrow 1 and 3 correspond to the lysate of colony 1 and 3 respectively after expression induction by IPTG. Both lysate were probed with anti-poly-His-tag antibodies (His-probe Antibody (H-3)) followed by HRP-linked secondary antibody (m-IgGκ BP-HRP) incubation.

As a first approach for recombinant protein expression, we have chosen a prokaryotic system. Compared with other expression systems, *E. coli* serves as an excellent host for recombinant protein production because it provides an economical and fast way to produce recombinant proteins in relatively large amounts, although yields of correctly folded and functional protein can be low because of protein aggregation (Li and Li, 2009; Li et al., 2015). The expression of recombinant plant subtilases in prokaryotic systems was already shown by several research groups. Li and co-authors (2015), reported a successful production of the recombinant AtSBT1.9 subtilase tagged by maltose binding protein (MBP). They used *E. coli* DH5α and vector pMD18-T for DNA manipulation and *E. coli* BL21(DE3) and vector pMALc2x for expression (Li et al., 2015). *E. coli* DH5α has the same mode of action as our *E. coli* One Shot TOP10 for DNA manipulation and we also used *E. coli* BL21-Codon plus. The major difference between VviSBT4.19 and AtSBT1.9 is protein solubility. While AtSBT1.9 is a soluble protein (Li et al., 2015), the ccSOLomics software (version 2012) predicted that our VviSBT4.19 had 3% of solubility propensity, which means that it is a insoluble protein, thus different expression induction strategies may have to be used.

Plattner and co-workers reported successful production of the barley subtilase BAJ93208 in *E. coli*. They have used an expression system called *E. coli* SHuffle expression system and the expression vector pJOE-SP-MCS without signal peptide. The promoter of the pJOE vectors is very tightly regulated, so they suspect that it might have been the reason for success (Plattner et al., 2014). It may be a good approach to try in our experiments in order express our protein because this method is mentioned to be good in insoluble protein expression as well (Lobstein et al., 2012).

As other possible strategy to produce the recombinant grapevine VviSBT4.19 is the use of a eukaryotic system already described for the production of plant subtilases, e.g insect cells when expressing LeSBT1 (Janzik et al., 2000), and tomato suspension culture system, while expressing LeSBT3 (Cedzich et al., 2009).

5. Conclusion

Previous studies have shown that 13 grapevine subtilases were putatively involved in the defense mechanism against the downy mildew causal agent. In the present work, those subtilases were compared to other subtilases previously described as participating in plant immunity. Based on a phylogenetic analysis, seven grapevine subtilases were selected, that closely correlated to *Arabidopsis* and cotton subtilases involved in plant defence against pathogen infection. Their expression profile was evaluated in both compatible and incompatible grapevine- *P. viticola* interactions and either jasmonic acid or salicylic acid elicitation. When comparing resistant and susceptible grapevine genotypes, two of the selected subtilases (VviSBT5.3a and VviSBT4.19) increase their expression in response to *P. viticola* inoculation and JA and SA stimulus in the resistant genotype, Regent. These findings confirmed the involvement of these two potential candidates in grapevine immunity. Thus, to understand their model of action may uncover new disease control strategies.

We have chosen VviSBT4.19 for further characterization and performed the recombinant protein production. To produce the recombinant VviSBT4.19, the gene's open reading frame was isolated and several cloning steps were performed to clone it both in cloning and expression vectors. At all of the cloning steps we have confirmed sequence insertion and the gene sequence. We have faced several constrains in the induction of the recombinant protein expression and at the present, we are trying to optimise the expression and western blot conditions to overcome these problems, concerning the protein production and interaction between antibodies and our fusion protein in order to proceed with protein purification, identification by mass spectrometry and the measurement of enzyme activity.

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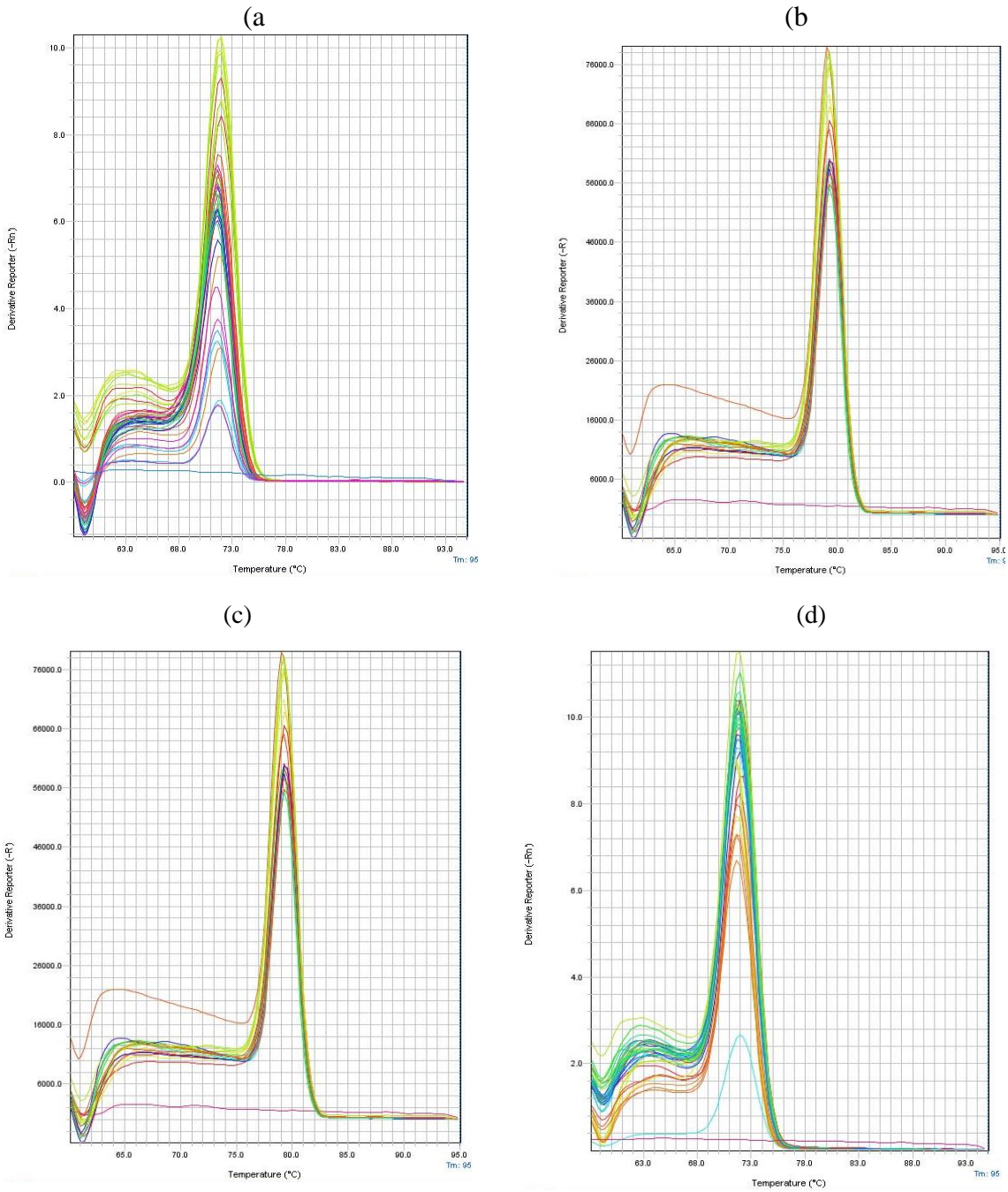
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7. Appendix
Supplementary data 1: Melting curve of targeted genes



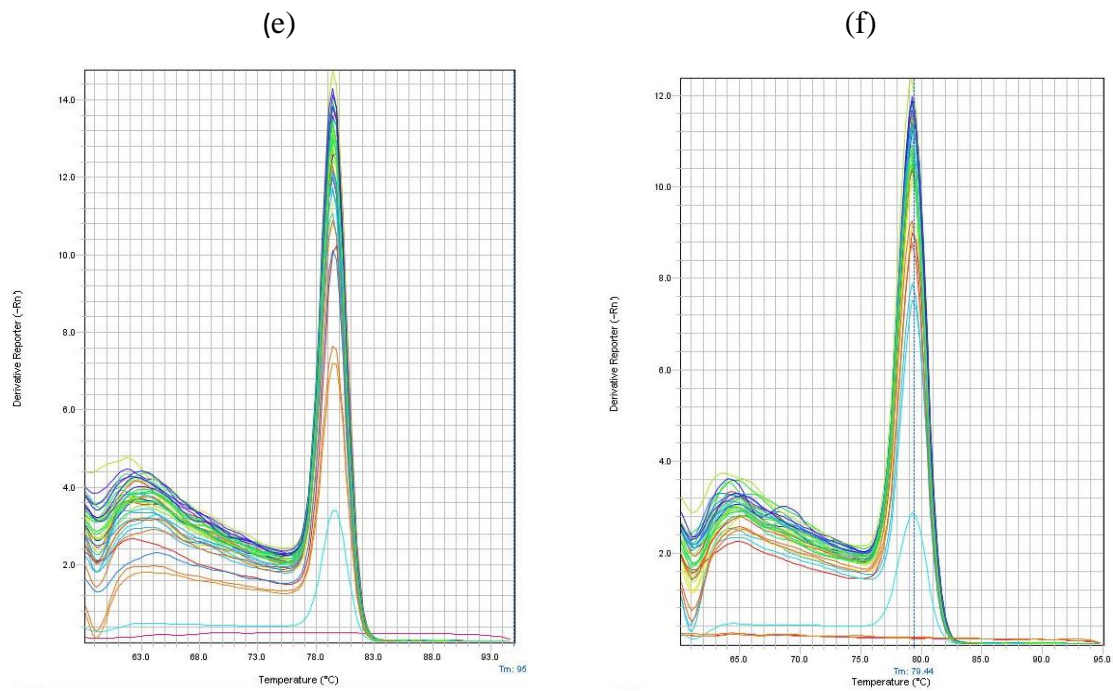


Figure 11: Melting curve of targeted genes, performed in StepOne™ Real-Time PCR system (Applied Biosystems, Sourceforge, USA). Elicitation: VviSBT4.19 Isoform X1 (a), VviSBT3.21 Isoform X1 (b), VviSBT5.3a (c). Inoculation: VviSBT4.19 Isoform X1 (d), VviSBT5.3a (e), VviSBT3.21 Isoform X1 (f).

Supplementary data 2:

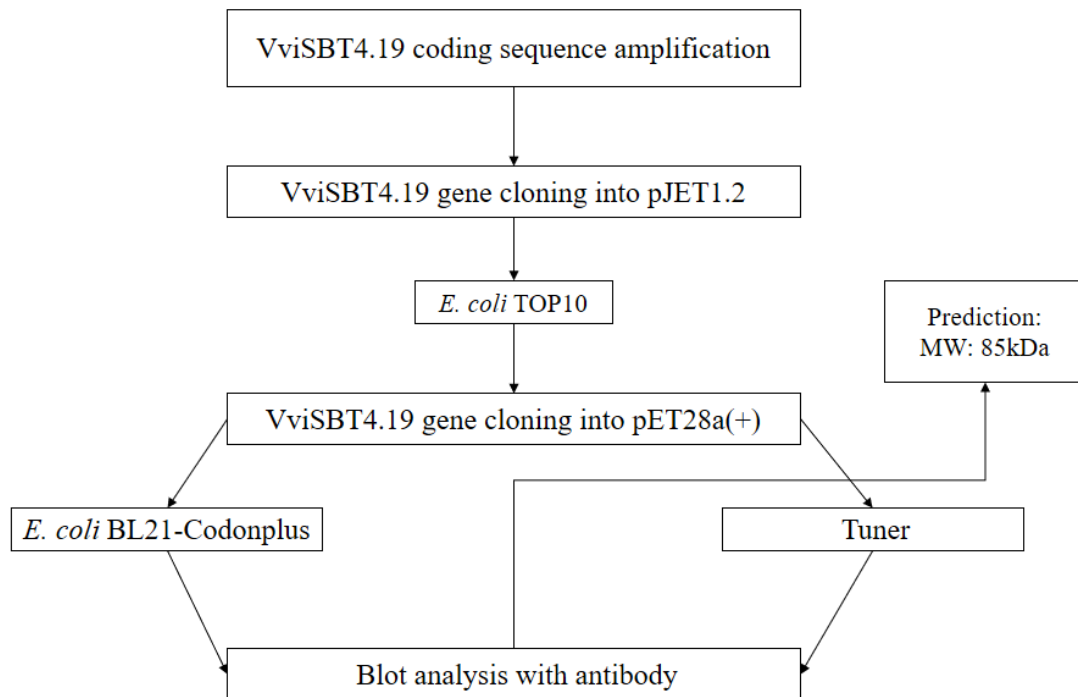


Figure 12: VviSBT4.19 cloning and expression steps.

Supplementary data 3:

>VviSTB4.19 isoform X1

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AGCACAAACATTTTACCTAATTTAAGATCTTCAATCTTATTCA
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Figure 13: VviSBT4.19 coding sequence. The grey sequence represents the VviSBT4.19 Open reading frame. The yellow sequence fragments represent forward and reverse primers.